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Enzyme-controlled hygroscopicity and proton dynamics in sea cucumber (*Stichopus japonicus*) ovum peptide powders

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ABSTRACT

The enzyme-controlled hygroscopicity of peptide powders during storage at room temperature may have a profound effect on their properties. The present study aims to elucidate hygroscopicity, proton dynamics, as well as effect on the microstructure of sea cucumber ovum peptides (SCOPs) powder produced with different enzymes during storage. The SCOPs produced with Alcalase exhibited the strongest moisture absorption capacity, which was significantly higher than those of SCOPs produced with papain, neutrase, and trypsin ($P < .05$). This might be attributed to the greater hydrolysis of Alcalase and producing more polar groups. Moreover, the proton dynamic and the transformations of water populations varied greatly among the SCOPs with different enzymes. Even so, the SCOPs exhibited a common water migration rule that free water was gradually transformed into immobilized water that was simultaneously converted into bound water after moisture absorption. The hygroscopicity induced morphological changes of SCOPs, which was converted from a smooth amorphous structure into different sizes of particle agglomerates. Moreover, the SCOPs produced with Alcalase displayed more and smaller agglomerates than those produced with papain, neutrase, and trypsin. This study provides a theoretical basis for quality assurance of peptide powders, particularly those produced with Alcalase.

1. Introduction

Sea cucumber is a benthic marine organism distributed worldwide and used as food with high nutritional value in several Asian countries. It contains a higher level of protein and a lower level of fat than most other foods (Ye, Caihong, Yayan, Zhang, & Meitian, 2017). These abundant proteins are a rich source for making high quality bioactive peptides which have the potential to ameliorate disease or promote health (Olivera-Castillo et al., 2018). The aquaculture of sea cucumber has increased rapidly in Asia in recent decades (Anderson, Mills, Reg, & Lotze, 2011), and the total output of sea cucumber has reached > 204,000 tons in China (Fishery Bureau of the Ministry of Agriculture, 2016). In general, sea cucumber ova are deemed as low-value by-products generated during industrial processing, and remain to be fully utilized as value-added materials. The defatted sea cucumber ova are rich in protein and become a good source of bioactive peptides with a variety of functional properties, including antioxidant, calcium-binding and iron-binding activities (Sun et al., 2017; Sun et al., 2017). Therefore, sea cucumber ovum peptides might have a potential application in the development of novel functional foods. However, under normal conditions, the peptide powders possess strong hygroscopicity, leading to inappropriate changes of quality, including bridging, agglomeration, and even liquefaction (Aguilera, Valle, & Karel, 1995). The hygroscopicity of the polymer directly affects the physical stability of solid dispersion (Fujimori et al., 2016). These inappropriate changes could frustrate their utilization in the functional food industry.

The hygroscopicity of peptide powders has a close relationship with hydrophilic amino acid residues. Lee et al. (2014) evaluated the functional properties of poly-γ-glutamic acid produced by *Bacillus subtilis* D7 and reported that poly-γ-glutamic acid exhibits water-holding capacity and hygroscopicity. Further, Xue, Cheng, Wang, and Lin (2017) investigated adsorbed water mobility and distribution of two soybean antioxidant pentapeptides, Ser-His-Cys-Met-Asn (SHCMN) and Ser-His-Glu-Cys-Asn (SHECN) powder and revealed that the hygroscopicity of SHECN is greater than that of SHCMN. This might be attributed to the differences of chemical structure and amino acid composition - more

Abbreviations: SCOPs, Sea cucumber ovum peptides; ANOVA, Analysis of variance; SD, Standard deviation; RH, Relative humidity; DVS, Dynamic vapor sorption; EMC, Equilibrium moisture content; LF-NMR, Low field-nuclear magnetic resonance; CPMG, Carr–Purcell–Meiboom–Gill; SW, Spectral width; NS, Scan repetitions; NECH, Echo count; TW, Waiting time; FTIR, Fourier transform infrared; SEM, Scanning electron microscopy

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specifically, the SHECN has a Glu residue containing carboxyl group, which has a strong interaction with water molecule; however, the SHCMN has a Met residue containing –S–CH_3 in the side chain which has a weaker interaction with water molecule when compared to carboxyl group. Moreover, Lin, Yang, Li, Feng, and Zhang (2016) found that the dynamics of water mobility and distribution in soybean antioxidant peptide powders (SAPPs) with three different molecular weights (< 1 kDa, 1–3 kDa, and 3–10 kDa) varied under the same conditions, and that the moisture absorption capacity of SAPPs with a MW of < 1 kDa was the strongest. In contrast to the other two samples, SAPPs with a MW of < 1 kDa comprised shorter amino acid chains. Hydrolysis of peptide bonds would produce hydrophilic polar groups, including amino and carboxyl groups, which might increase the binding force between the water molecules and peptide interface (Fernández & Wider, 2003; Gaudichet-Maurin, Thominette, & Verdu, 2010; Martin, 2015).

At present, there are three producing methods successfully used for biological production enrichment of marine peptides, including solvent extraction, enzymatic hydrolysis, and microbial fermentation. Enzymatic hydrolysis is used as the preferred method due to absence of residual toxic chemicals and organic solvents in termination products (Li, Shang, Jiang, Zhang, & Su, 2016). Protein hydrolysis increases the number of ionizable groups and can expose hydrophobic groups, which can change physical or chemical environmental interactions (Jung, Murphy, & Johnson, 2010; Villanueva et al., 1999), including the interaction with water molecules. However, enzymes, presenting substrate specificity, determine the position at which the enzyme will catalyze peptide bond hydrolysis. For instance, papain is preference for cleaving an amino acid bearing a large hydrophobic side chain at the P2 position, while trypsin facilitates preferential release of N-terminal Arg and Lys at P1 position (Tavano, 2013). In addition, Alcalase is an endoproteinase with broad specificity and has been previously reported to cleave the peptide bonds at Gln-His, Ser-His, Leu-Tyr, and Tyr-Thr (Ottesen & Svendsen, 1970). Thus different hydrolytic actions of the enzymes not only result in different hydrolysis degree but also produce different amino acid profile of the hydrolysates.

To date, the moisture absorption process of peptides produced with different enzymes has not been investigated. Therefore, the present study aims to elucidate hygroscopicity and proton dynamics of sea cucumber ovum peptides (SCOPs) powder produced with different enzymes during storage by using dynamic vapor sorption and low-field nuclear magnetic resonance. Further, the effect of enzyme-controlled hygroscopicity on the primary structure and microstructure of the SCOPs powders was also be investigated. This study provides a theoretical basis for quality assurance of peptide powders.

2. Materials and methods

2.1. Materials and regents

Sea cucumber (*Stichopus japonicus*) ova were purchased from the Shangpintang Marine Biology Co., Ltd. (Dalian, China). Alcalase 2.4 L and neutrase 2.4 L were donated by Novozyme (Bagsvaerd, Denmark). Trypsin (from porcine pancreas) and papain (a cysteine protease from papaya latex) were bought from Bio Basic Inc. (Toronto, Canada) and Sangon Biotech Co. Ltd. (Shanghai, China), respectively.

2.2. Preparation of sea cucumber ovum peptides

The sea cucumber ovum peptides (SCOPs) were produced with trypsin, neutrase, papain, or Alcalase according to the method from the previous studies of Sun, Cui, Lin, et al. (2017). Sea cucumber ova were defatted with hexane/ethanol (3:1 v/v) at 50 °C for 6 h, filtered and naturally air-dried at room temperature. The defatted sea cucumber ova powder was mixed with Milli-Q (Millipore, Bedford, MA) water at a substrate concentration of 2% (*w/v*). The mixture was heated at 95 °C for 10 min to denature native proteins, and hydrolyzed by trypsin (37 °C, pH 8.0), neutrase (50 °C, pH 7.0), papain (50 °C, pH 7.0), and Alcalase (50 °C, pH 8.5) at a dose of 3000 U/g protein for 3 h. The hydrolysis reaction was terminated by heating the mixture at 95 °C for 10 min. The suspension was centrifuged at 12,000 × *g* for 20 min at 4 °C and the precipitate was discarded. The four kinds of supernatant was freeze-dried at −50 °C for 72 h and stored at −20 °C for further research.

2.3. Dynamic vapor sorption (DVS) measurements

Water sorption kinetics of the SCOPs was measured according to the method modified from Young, Edge, Staniforth, Steele, and Price (2005). In this study a humidity-controlled microbalance system (DVS apparatus, Surface Measurement System Ltd., London, U.K.) was used to determine the water sorption kinetics of SCOPs powders at a constant temperature of 25 °C by exposing powder samples to different relative humidity (RH) conditions. A preset parameter ranged from 0% to 90% at a 10% RH change gradient, from 90% to 95% with a span of 5% RH, and then in reverse order to 0% RH. Approximately 5 mg of SCOPs powders was placed on the aluminum holder, connected to a microbalance by a hanging wire. The aluminum holder was located in a thermostatically controlled chamber with a constant dry nitrogen gas flow mixed with water vapor flow for controlling the demanded RH. The total gas flow rate was 500 mL/min. First of all, the desired RH and the equilibrium criterion were set. Then each sample was dried at 25 °C and further equilibrated to 0% RH in the DVS chamber for 3 h. Subsequently, the RH was increased to the target value and then in reverse order to 0% RH to allow water uptake and structural collapse. The equilibrium criterion was that a change in mass over time (dm/dt) was lower than 0.002% per minute during 5 min consecutively or that the maintenance time was over 360 min. The sample mass, target RH, the running time, and actual vapor pressure were automatically collected throughout the isotherm run every 60 s. The experiment was carried out in triplicate.

2.4. Low-field nuclear magnetic resonance (LF-NMR) measurements

Based on the above measurements, a representative condition (25 °C, 80% RH) was chosen to perform the LF-NMR measurements by using a 21 MHz MesoQMR23-060 h NMR analyzer (Niumag Analytical Instrument Co., Shanghai, China). Approximately 100 mg of SCOPs was placed into 15 mm NMR glass tubes with a hydrogen-free sealing tape. To maintain a constant temperature and humidity, the unsealed glass tubes with SCOPs were numbered and placed into a constant temperature incubator for 40 h. The instrument parameters were set at 25 °C with a RH of 80%. At different storage times (from 0 h to 40 h), the mass of the samples was weighed and T_2 relaxation times were measured using the Carr-Purcell-Meiboom-Gill (CPMG) sequence. The T_2 measurement parameters were set to a τ-value of 100 μs (echo time between 90° and 180° pulses), the spectral width (SW) of 200 KHz, scan repetitions (NS) of 128, echo count (NECH) of 5000, and waiting time (TW) of 500 ms. The experiment was carried out in triplicate. The analysis of LF-NMR T_2 relaxation data and the fitting of distributed exponential curve were performed as described by Yang et al. (2016). A linear distribution of exponential decays was defined in the following equation:

$$M(t) = \sum_{i=1}^{n} M_{2i} \exp\left(\frac{-t}{T_{2i}}\right) + e(t) \quad (1)$$

Where $M(t)$ is the residual magnetization at a given time t, n is the number of exponential components in the samples, M_{2i} is relaxation amplitude of the i_{th} component, T_{2i} is transverse relaxation time of the i_{th} component, and $e(t)$ is the residual error. Three parallel samples of different storage times were measured.